evolution

A Centre of Excellence for Civil Service Examination Guidance

B 11, Second Floor, Commercial Complex, Batra Cinema Road
Next to ICICI A.T.M., Dr. Mukherjee Nagar, DELHI - 110 009
Phone: 011 - 32974645, 32974651, 47092329

Botany Paper II -

Biotechnology

Table of contents

Syllabus

Biotechnology (Botany Optional)

PART 1: Genetic engineering

1. Methods of transfer of genes
2. Transgenic crops
3. Bio-safety aspects

PART 2: Tools and techniques

1. DNA sequencing
2. Probe
3. Southern blotting
4. DNA fingerprinting
5. PCR
6. FISH

DNA Sequencing

An introduction to DNA sequencing

DNA is the information store that ultimately dictates the structure of every gene product and governs every part of the organisms. The order of the bases along DNA contains the complete set of instructions that make up the genetic inheritance.

The term DNA sequencing refers to methods for determining the order of the nucleotides bases adenine, guanine, cytosine and thymine in a molecule of DNA.

DNA sequencing is very significant in research and forensic science. The main objective of DNA sequence generation method is to evaluate the sequencing with very high accuracy and reliability.

By knowing the DNA sequence, the cause of the various diseases can be known. We can determine the sequence responsible for various diseases and can be treated with the help of Gene therapy.

Benchmarks in DNA sequencing

- 1953 Discovery of the structure of the DNA double helix.
- 1972 Development of recombinant DNA technology, which permits isolation of defined fragments of DNA; prior to this, the only accessible samples for sequencing were from bacteriophage or virus DNA.
- 1975 The first complete DNA genome to be sequenced is that of bacteriophage φX174
- 1977 Allan Maxam and Walter Gilbert publish "DNA sequencing by chemical degradation". Fred Sanger, independently, publishes "DNA sequencing by enzymatic synthesis".
- 1980 Fred Sanger and Wally Gilbert receive the Nobel Prize in ChemistryGilbert and Maxam method

Maxam – Gilbert method of sequencing

In 1976-1977, Allan Maxam and Walter Gilbert developed a DNA sequencing method based on chemical modification of DNA and subsequent cleavage at specific bases.

The method requires radioactive labelling at one end and purification of the DNA fragment to be sequenced. Chemical treatment generates breaks at a small proportions of one or two of the four nucleotide based in each of four reactions (G,A+G, C, C+T). Thus a series of labelled fragments is generated, from the radiolabelled end to the first 'cut' site in each molecule.

The fragments in the four reactions are arranged side by side in gel electrophoresis for size separation. To visualize the fragments, the gel is exposed to X-ray film for autoradiography, yielding a series of dark bands each corresponding to a radiolabelled DNA fragment, from which the sequence may be inferred.

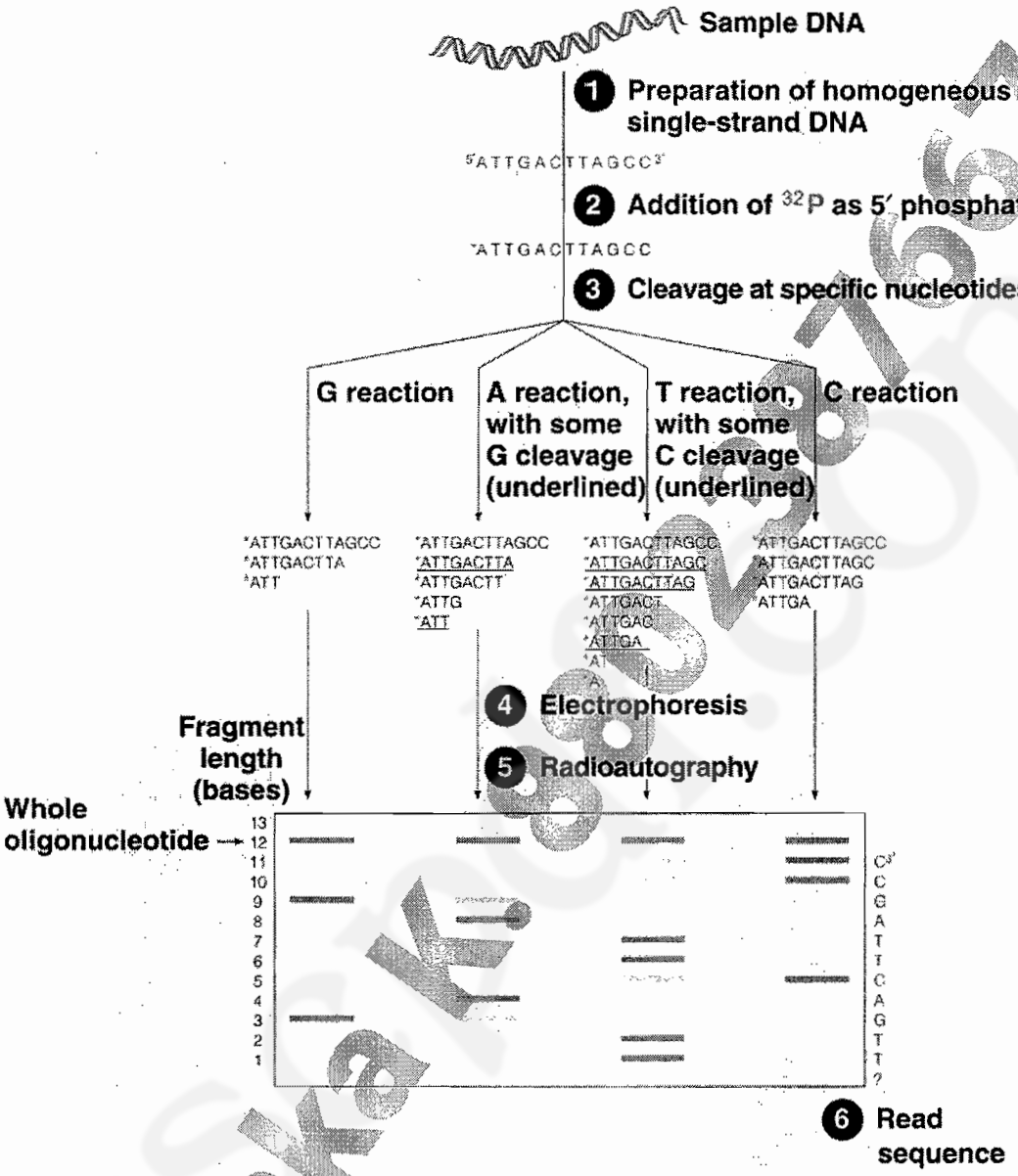


Figure: Maxam Gilbert Method

Stages:

1. Double-stranded DNA to be sequenced is labelled by attaching a radioactive phosphorus (^{32}P) group to the 5' end. Polynucleotide kinase enzyme and ^{32}P-dATP is used here.
2. Using dimethyl sulphoxide and heating to 90°C, the two strands of the DNA are separated and purified (*e.g.* using gel electrophoresis and the principle that one of the strands is likely to be heavier than the other due to the fact that it contains more purine nucleotides (A and G) than pyrimidines (C and T) which are lighter).
3. Single-stranded sample is split into separate samples and each is treated with one of the cleavage reagents. This part of the process involves **alteration** of bases (*e.g.* dimethylsulphate methylates guanine) followed by **removal** of altered bases. Lastly, piperidine is used for **cleavage** of the strand at the points where bases are missing.

Base specificity	Chemical used for base alteration	Chemical used for altered base removal	Chemical used for strand cleavage
G	Dimethylsulphate	Piperidine	Piperidine
A+G	Acid	Acid	Piperidine
C+T	Hydrazine	Piperidine	Piperidine
C	Hydrazine + alkali	Piperidine	Piperidine
A>C	Alkali	Piperidine	Piperidine

- If reactions have been arranged to give only one, or a few, cleavages per DNA molecule, a nested set of end-labelled DNA fragments of different lengths is produced.
- The samples are run together on a sequencing gel which separates the fragments by electrophoresis depending on their size. DNA bands in the gel are visualized by autoradiography (^{32}P-labelled 5' end fogs photographic film). The DNA sequence is read directly from the gel.

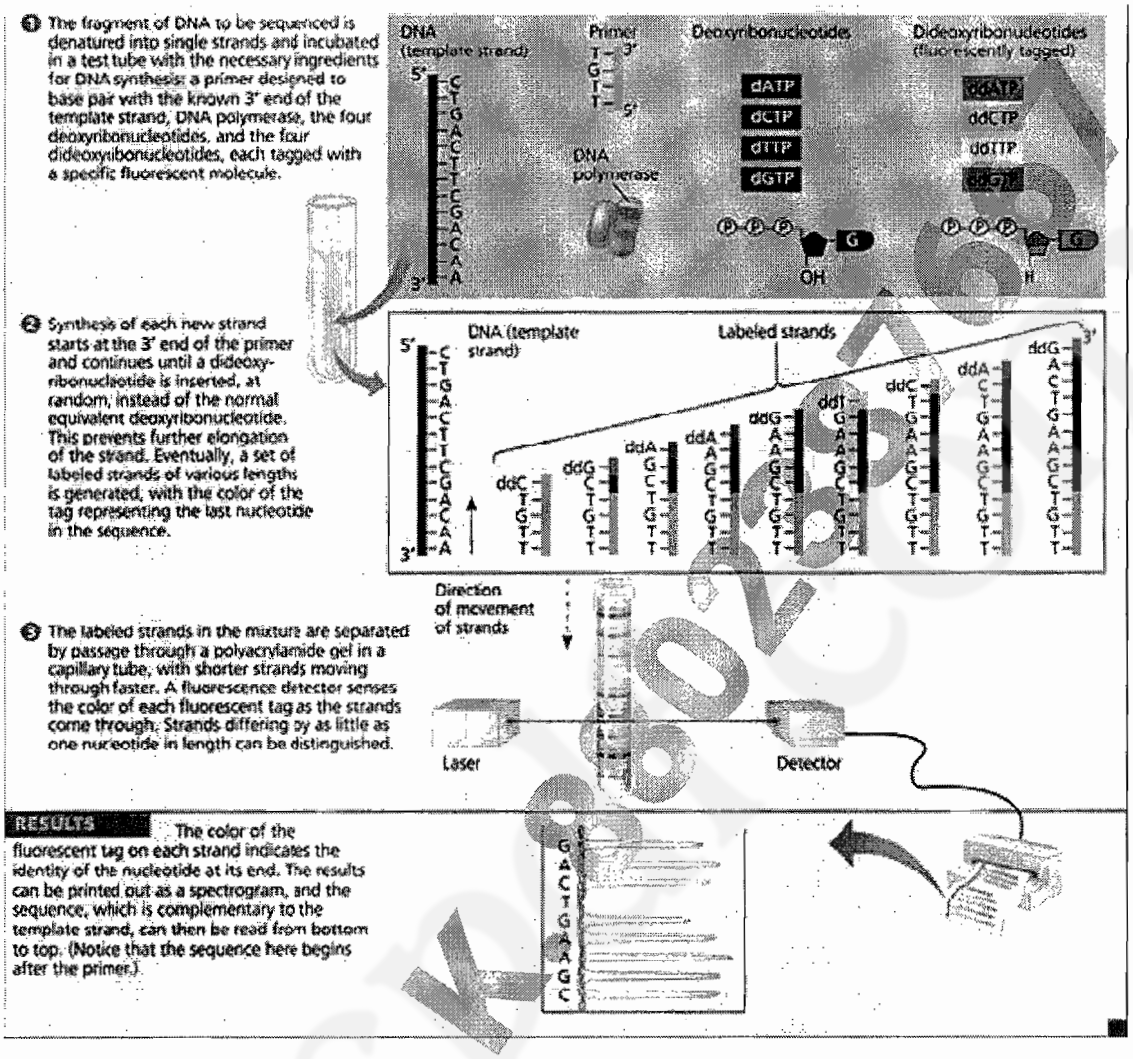
Sanger's chain-termination method of sequencing

Chain-termination method is more efficient and uses fewer toxic chemicals and lower amount of radioactivity than the method of Maxam and Gilbert.

The key principle of the Sanger method was the use of dideoxynucleotide triphosphates (ddNTPs) as DNA chain terminators.

The chain termination method requires a single-stranded DNA template, a DNA primer,a DNA polymerase, radioactively or fluorescently labelled nucleotides, and modified nucleotides that terminate DNA strand elongation. The DNA sample is divided into four separate sequencing reactions, containing all four of the standard deoxynucleotides(dATP, dGTP, dCTP, dTTP) and the DNA polymerase. To each reaction is added only one of the four dideoxynucleotide (ddATP, ddGTP, ddCTP, ddTTP) which are the chain terminating nucleotides, lacking a 3'-OH group required for the formation of a phosphodiester bond between two nucleotides, thus terminating DNA strand extension and resulting in DNA fragments of varying length.

The newly synthesized and labelled DNA fragments are heat denatured , and separated by size by gel electrophoresis on a denaturing polyacrylamide-urea gel with each of the four reactions run in one of the four individual lanes(lanes A, T, G,C), the DNA bands are then visualized by autoradiography or UV light, and the DNA sequence can be directly read off the X-ray film or gel image. A dark band in a lane indicates a DNA fragment that is result of chain termination after incorporation of a dideoxynucleotide (ddATP, ddGTP, ddCTP, or ddTTP). The relative position of the different bands among the four lanes are then used to read (from bottom to top) the DNA sequence.



The technical variations of chain termination sequencing include tagging with nucleotides containing radioactive phosphorus for labelling, or using a primer labelled at the 5' end with a fluorescent dye. Dye- primer sequencing facilitates reading in an optical system for faster and more economical analysis and automation.

Chain termination methods have greatly simplified DNA sequencing. Limitations include non-specific binding of the primer to the DNA, affecting accurate read-out of the DNA sequence,and DNA secondary structures affecting the fidelity of the sequence.

Large-scale sequencing strategies

Current methods can directly sequence only relative short (300-1000 nucleotides long) DNA fragments in a single reaction. The main obstacle to sequencing DNA fragments above this size limit is insufficient power of separation for resolving large DNA fragments that differ in length by only one nucleotide.

Large scale sequencing aims at sequencing very long DNA pieces, such as whole chromosomes. It consist of cutting (with restriction enzymes) or shearing (with mechanical forces) large DNA

fragments into shorter DNA fragments. The fragmented DNA is cloned into a DNA vector, and amplified in *E.coli*. Short DNA fragments purified from individual bacterial colonies are individually sequenced and assembled electronically into one long, contiguous sequence. This method does not require any pre- existing information about the sequence of the DNA and is reffered to as de novo sequencing. Gaps in the assembled sequence may be filled by primer walking.

Applications

Some important applications of DNA sequencing are:

1. To analyse any protein structure and function we must have the knowledge of its primary structure i.e its DNA sequence.
2. With its study we can understand the function of a specific sequence and the sequence responsible for any disease.
3. With the help of comparative DNA sequence study we can detect any mutation.
4. Kinship study.
5. DNA fingerprinting.
6. By knowing the whole genome sequence, Human genome project get completed.

Probes

The isolation of specific DNA sequences from a tissue sample was a challenge for early molecular biologists. Unlike proteins, which have diverse sizes and charges, there is no easy way separate out a specific sequence of DNA based on its general physical and chemical properties. One cannot chemically stain for a specific sequence of DNA. In contrast, it is often possible to use a specific stain to detect a specific protein. However, once a DNA sequence is known, the complementary strand can be synthesized and used to detect the strand of interest in a sample.

Gene probe is single-stranded DNA or RNA fragment used in genetic engineering to search for a particular gene or other DNA sequence. The probe has a base sequence complementary to the target sequence and will thus attach to it by base pairing.

Types of probes

There are essentially four types of probes.

1. **Oligonucleotide probes:** These are produced synthetically by an automated chemical synthesis. The method utilizes readily available deoxynucleotides which are economical, but it requires that the investigator knows the specific nucleotide sequence. Designing the sequence of the probe is one of the more critical decisions required when using oligonucleotide probes. These probes have the advantages of being resistant to RNases and are small, generally around 40-50 base-pairs. This is ideal for *in situ* hybridization because their small size allows for easy penetration into the cells or tissue of interest.

2. **Single stranded DNA probes:** These have similar advantages to the oligonucleotide probes except they are much larger, probably in the 200-500 bp size range. They can be produced by reverse transcription of RNA or by amplified primer extension of a PCR-generated fragment in the presence of a single antisense primer.

3. **Double stranded DNA probes:** These can be produced by the inclusion of the sequence of interest in a bacterium which is replicated, lysed and the DNA extracted, purified and the sequence of interest is excised with restriction enzymes. The advantage of the bacterial preparation is that it is possible to obtain large quantities of the probe sequence. Because the probe is double stranded, it means that denaturation has to be carried out prior to hybridization. These probes are generally less sensitive because of the tendency of the DNA strands to rehybridize to each other and are not as widely used today.

4. **RNA probes (cRNA probes or riboprobes):** RNA probes have the advantage that RNA-RNA hybrids are very thermostable and are resistant to digestion by RNases. This allows the possibility of post-hybridization digestion with RNase to remove non-hybridized RNA.

Using probes

To detect hybridization of the probe to its target sequence, the probe is tagged (or "labeled") with a molecular marker of either radioactive or fluorescent molecules. Commonly used markers are ^{32}P or Digoxigenin, which is a non-radioactive, antibody-based marker.

DNA sequences or RNA transcripts that have moderate to high sequence similarity to the probe are then detected by visualizing the hybridized probe via autoradiography or other imaging techniques. Normally, either X-ray pictures are taken of the filter, or the filter is placed under UV light.

The following types of hybridization methods are common.

1. **Normal hybridization**. Normal hybridization requires the isolation of DNA or RNA, separating it on a gel, blotting it onto nitrocellulose and probing it with a complementary sequence.
2. **In situ hybridization**. *In situ* hybridization is a method of localizing and detecting specific mRNA sequences in morphologically preserved tissues sections or cell preparations by hybridizing the complementary strand of a nucleotide probe to the sequence of interest.
3. **DNA microarray**. A DNA microarray (also commonly known as DNA chip or biochip) is a collection of microscopic DNA spots attached to a solid surface. Each DNA spot contains picomoles (10−12 moles) of a specific DNA probes. These can be a short section of a gene or other DNA element that are used to hybridize a cDNA or cRNA. Scientists use DNA microarrays to measure the expression levels of large numbers of genes simultaneously.

Applications

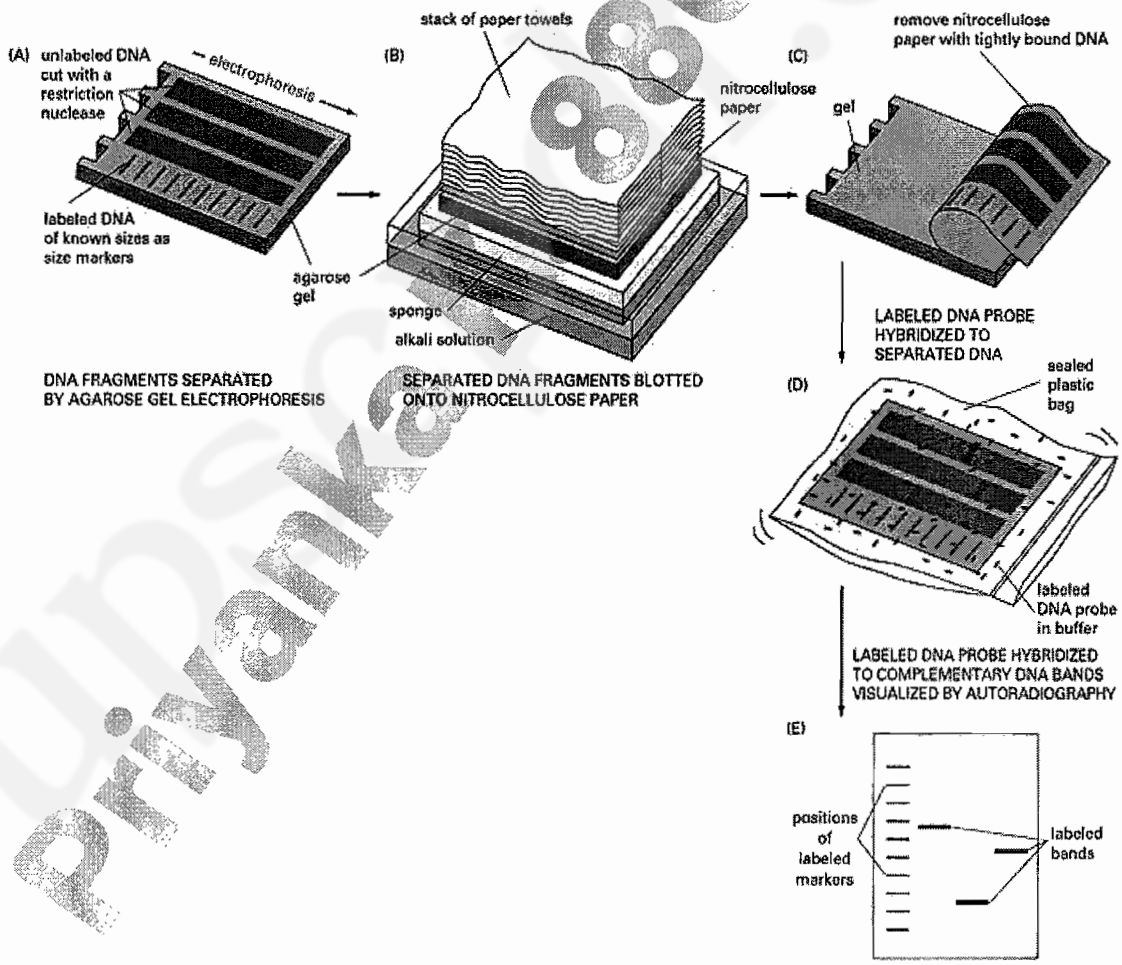
- **Applications in Medical Research**. There are at least three basic applications of nucleic acid probes in medical research: (1) detection of pathogenic microorganisms, (2) detection of changes to nucleic acid sequences, and (3) detection of tandem repeat sequences.
- **Use in forensic science**. In forensic science, hybridization probes are used for detection of short tandem repeats (microsatellite) regions and in restriction fragment length polymorphism (RFLP) methods, all of which are widely used as part of DNA profiling analysis.
- **Uses in Microbial Ecology**. Within the field of microbial ecology, oligonucleotide probes are used in order to determine the presence of microbial species, genera, or microorganisms classified on a more broad level, such as bacteria, archaea, and eukaryotes via fluorescence in situ hybridization (FISH).

Southern blotting & DNA Fingerprinting

Southern blotting

A **Southern blot** is a method used in molecular biology for detection of a specific DNA sequence in DNA samples. **Southern blotting** combines transfer of electrophoresis-separated DNA fragments to a filter membrane and subsequent fragment detection by probe hybridization.

E. M. Southern (1975) first described a method for the transfer of DNA restriction fragments from agarose gels to cellulose nitrate (nitrocellulose) membranes and then hybridization of the resulting blot-immobilized DNA to radioactively labeled DNA probes. Since then, the term "Southern blot" has been used to describe any hybridization of DNA on blots to DNA probes, regardless of the type of membrane used. [Northern blot refers to RNA/DNA blot hybridizations, Western blots are protein/ antibody hybridizations.]



Steps

1. The mixture of double-stranded DNA fragments generated by restriction nuclease treatment of DNA is separated according to length by electrophoresis.
2. A sheet of either nitrocellulose paper or nylon paper is laid over the gel, and the separated DNA fragments are transferred to the sheet by blotting. The gel is supported on a layer of sponge in a bath of alkali solution, and the buffer is sucked through the gel and the nitrocellulose paper by paper towels stacked on top of the nitrocellulose. As the buffer is sucked through, it denatures the DNA and transfers the single-stranded fragments from the gel to the surface of the nitrocellulose sheet, where they adhere firmly. This transfer is necessary to keep the DNA firmly in place while the hybridization procedure (step D) is carried out.
3. The nitrocellulose sheet is carefully peeled off the gel.
4. The sheet containing the bound single-stranded DNA fragments is placed in a sealed plastic bag together with buffer containing a radioactively labeled DNA probe specific for the required DNA sequence. The sheet is exposed for a prolonged period to the probe under conditions favoring hybridization.
5. The sheet is removed from the bag and washed thoroughly, so that only probe molecules that have hybridized to the DNA on the paper remain attached. After autoradiography, the DNA that has hybridized to the labeled probe will show up as bands on the autoradiograph.

DNA Fingerprinting

Introduction

DNA is the genetic material found within the cell of all organisms. With the exception of identical (monozygotic) twins at birth, the complete DNA of each individual is unique.

DNA Fingerprinting is a method of establishing identification that compares fragments of deoxyribonucleic acid (DNA) between two sources. It is sometimes called ***DNA Typing*** or ***DNA Profiling***. In other words, it is a technique, by which an individual can be identified at molecular level. This remarkable technology provides exclusion as well as positive identification with virtually 100% precision.

DNA fingerprinting was first developed as an identification technique in 1985 by ***Sir Alec Jeffery*** at the University of Leicester, UK. Originally used in criminal investigations, DNA fingerprinting soon came to be used in and forensic science and to detect the presence of genetic diseases. The first criminal conviction based on DNA evidence in the United States occurred in 1988.

Currently, DNA fingerprinting technology is applied to identify the source of biological samples found at scenes of crime, to resolve disputes of maternity /paternity, in identification of mutilated remains, in identification of rape/ murder accused, in identification of missing child, in exchange of babies in hospital wards, in forensic wildlife, in protection of farmers rights and biodiversity.

Protocol of DNA Fingerprinting

The basic design of a DNA Fingerprinting Experiment is relatively simple. Although it compares the fragments of DNA, it no where involves sequencing.

The major and classically established method of DNA Fingerprinting is ***Restricting Fragment Length Polymorphism*** (***RFLP***) ***Analysis***.

The steps in this approach are as follows.

A DNA fingerprint experiment is started by first extracting a DNA sample from body tissue or fluid such as hair, blood, or saliva.

The DNA sample is then segmented using a specific restriction enzyme. This restriction enzyme will generate DNA Fragments in a specific manner, depending on the distribution of the corresponding restriction sites on the sample DNA. It is important to note that even on the second sample, whose fingerprint pattern we have to compare with this DNA's fingerprint, is cleaved using the same restriction enzyme and under strictly the same conditions. Failure to do so leads to errors in the result.

The segments obtained from restriction enzyme treatment are arranged by size using the process of Agarose Gel Electrophoresis or Polyacrylamide Gel Electrophoresis (PAGE).

The arranged segments are then marked with probes and exposed on X-ray film, where they form a characteristic pattern of black bars. *This characteristic pattern of bands is called the DNA fingerprint.*

If the DNA fingerprints produced from two different samples match accurately band by band, the two samples came from the same person.

Why two different persons cannot have identical bands of DNA fragments after Southern Blotting?

Any restriction enzyme cuts a sample of DNA according to the restriction sites which are present on this DNA. Only the restriction sites can be identified and cut by the restriction enzyme.

There are hundreds of restriction sites for a particular restriction endonuclease in a person's genetic material, i.e. Genomic DNA. There are chances that at least some of these restriction sites are mutated in some other individual. If the restriction site is mutated, it can be bound and cleaved by the restriction endonuclease enzyme. Thus, the total number of fragments as well as the size of the fragments will vary from one person to another. Based on this difference, individual identification can be established. With the advent of certain modern approaches, this remarkable technology provides exclusion as well as positive identification with virtually 100% precision.

Other Approaches

PCR analysis

With the invention of polymerase chain reaction (PCR), DNA fingerprinting took huge strides forward in both discriminating power and ability to recover information from very small starting samples. PCR involves the amplification of specific regions of DNA using a cycling of temperature and a thermostable polymerase enzyme along with sequence specific primers of DNA.

Commercial kits that used single nucleotide polymorphisms (***SNPs***) for discrimination became available. These kits use PCR to amplify specific regions with known variations and hybridize them to probes anchored on cards, which results in a colored spot corresponding to the particular sequence variation.

One of the primary complaints against RFLP was that it was slow and required large quantities of DNA to be used. This led to the development of PCR-based methods which required smaller amounts of DNA. PCR analysis is quicker too plus it allows the analysis of DNA more degraded than those

used in RFLP analysis. It allows to determine a DNA profile for mixed samples, such as a vaginal swab from a sexual assault victim.

VNTR or STR Analysis

The most prevalent method of DNA fingerprinting used in modern labs is based on PCR and uses short tandem repeats (STR).

This method uses highly polymorphic regions that have short repeated sequences of DNA (the most common is 4 bases repeated, but there are lengths in use, including 3 and 5 bases). Because different people have different numbers of repeat units, these regions of DNA can be used to discriminate between individuals. These STR loci (locations) are targeted with sequence-specific primers and are amplified using PCR. The DNA fragments that result are then separated and detected using electrophoresis. There are two common methods of separation and detection, capillary electrophoresis (CE) and gel electrophoresis.

The polymorphisms arise due to Variable Number of Tandem Repeats or VNTR displayed at each STR region. They are by very common because of slipped strand mispairing during replication of repeated sequences.

The VNTRs can also be analyzed by arranging the cut segments according to length and then subjecting the fragments to Southern Blotting method. However, the PCR approach gives quicker results.

When looking at multiple loci, it is the unique combinations of these polymorphisms to an individual that makes this method discriminating as an identification tool. The more STR regions that are tested in an individual the more discriminating the test becomes.

From country to country different STR based DNA profiling systems are in use. In North America ***CODIS*** is prevalent, while in the UK the ***SGM+*** system, which is compatible with The National DNA Database is in use. Whichever system is used, many of the STR regions under test are the same. These DNA profiling systems are based around multiplex reactions, whereby many STR regions will be under test at the same time.

AmpFLP

Another technique, AmpFLP, or amplified fragment length polymorphism was also put into practice during the early 1990's. This technique was also faster than RFLP analysis and used PCR to amplify DNA samples. It relied on variable number tandem repeat (VNTR) polymorphisms to distinguish various alleles, which were separated on a polyacrylamide gel using an allelic ladder (as opposed to a molecular weight ladder). Bands could be visualized by silver staining the gel. One popular locus for fingerprinting was the D1S80 locus.

AmpFLP analysis can be highly automated, and allows for easy creation of phylogenetic trees based on comparing individual samples of DNA. Due to its relatively low cost and ease of set-up and operation, AmpFLP remains popular in lower income countries.

Application of DNA profiling and achievements in India

1. ***In Forensic investigation such as identification of mutilated body of the victims of some catastrophe, investigation of murder and sexual assault cases etc***. In such cases there must be a 100% DNA profile overlap between source and reference DNA to hold some one guilty of a violent crime or sexual assault.

   In identification of mutilated body, the profile may be matched to the children's or parents of the victim. In India DNA profiling was applied in cases like Rajiv Gandhi Murder, Beant Singh assassination and recently in news Naina Sahni and Priyadarshini Matoo cases.

2. ***Establishment of Parentage or Disputed Family relations or in finding lost children etc.*** Here the child's genetic profile is matched compared to proposed parent's genetic profile. The child's profile must correspond to half each of mother's and father's profile. However there is a rare possibility that some other couple together generate profile matching the child's profile.

3. ***DNA Based diagnosis*****:** The centre of DNA fingerprinting and diagnostics (CDFD) at Hyderabad has developed probes to identify the mutations leading to Huntington's disease, Thalassemia, Sickle Cell Anemia, Mental Retardation, Fragile-X Syndrome, Duchenne Muscular Dystrophy, Huntington's Disease, Azoospermia, etc. These probes help in prenatal diagnosis and Genetic Counselling.

4. ***Establishing Phylogenetic Relations*****:** CDFD has developed probes from *Bangarus fasciatus* (the banded Krait Snake) that reveals relationship among different groups of reptiles and also of Birds. This approach can be applied to a widerange of organisms using appropriate probes.

5. ***Verification of seed stocks before approving them for field trials or granting them patents.*** In 1997 a patent application moved by Pakistan for a variety of Basmati Rice was opposed by India on the grounds that latter has already had patented the same variety of Basmati Rice. India could successfully protect the patent for its own variety because DNA profiling showed that the two Basmati varieties are the same for which India already had the patent.

6. ***Wild lfie Conservation especially of Asiatic lions and tigers in India***: It was earlier proposed that population of these two animals in India has a very low level of Genetic variations due to high inbreeding and population bottleneck. However DNA profiling established that the genetic variability as heterozygosis exceeds 20% in these populations and it was about the same before the population bottleneck.

   Further, the individuals with high genetic variability has been identified which can be used for conservation breeding programme.

   Based on this contribution of the CDFD, a decision for setting up of a Centre for Wildlife Conservation in collaboration with the Nehru Zoological Park, Department of Forests, Government of Andhra Pradesh; Centre for Cellular and Molecular Biology; Centre for DNA Fingerprinting and Diagnostics; Central Zoo Authority of India; Department of Biotechnology, has been taken. This Centre will be set up close to the Nehru Zoological Park, Hyderabad.

7. ***Isolation of Genotypes for useful characters*** among silk worms and Honey bees which can electively be cultured for high qualify yield.

8. ***A comparative study of Y-chromosome*** by use of DNA profiling gives information on population migration that have been taken place in older times.

9. ***In easy detection of crime***. In Britain, National DNA DATA Base has been developed which has the DNA database of a large number of people. It helps the police to track down the culprits in a very efficient manner. Similar steps have been taken in India too. The Department of Biotechnology under the Ministry of Science and Technology released the Draft Human DNA Profiling Bill, 2015 on August 5, 2015. The Draft Bill regulates the use of DNA analysis. It establishes national, state and regional level DNA data banks and the DNA Profiling Board.

Limitations

1. Error during the lab process can increase inaccuracy manifold.
2. Damaged DNA may give inaccurate bands
3. Some segments may be missing from the damaged DNA which gives the impression of homozygosis.
4. Can't be applied on Identical twins
5. In some cases, DNA samples have been planted at the site of crime to falsely implicate people

PCR

PCR (polymerase chain reaction) is a key technique in molecular genetics that permits to amplify a precise fragment of DNA in almost any number of copies.

PCR was invented by *Kary Mullis* in 1983.

Basic Components of PCR

A modern polymerase chain reaction requires six basic components to work:

1. *the DNA segment* to be copied,
2. *primers* to delimit the segment,
3. *Taq polymerase* to do the copying,
4. *DNA nucleotides* to serve as feedstock,
5. a *chemical buffer environment*, and
6. a machine called a *thermal cycler*.

The thermal cycler holds multiple test tubes with multiple polymerase chain reactions, each holding 15 to 100 microliters. About a hundred nanograms of DNA base are used.

The standard PCR reaction: Principle and procedure

PCR is a rapid and versatile *in vitro* method for amplifying defined target DNA sequences present within a source of DNA.

Usually, the method is designed to permit *selective amplification* of a specific target DNA sequence(s). To permit such selective amplification, some prior DNA sequence information from the target sequences is required. This information is used to design *two oligonucleotide primers* (*amplimers*) which are specific for the target sequence and which are often about 15–25 nucleotides long.

After the primers are added to *denatured template DNA*, they bind specifically to complementary DNA sequences at the target site. In the presence of a suitably heat-*stable DNA polymerase and DNA precursors* (the four deoxynucleoside triphosphates, dATP, dCTP, dGTP and dTTP), they initiate the synthesis of new DNA strands which are complementary to the individual DNA strands of the target DNA segment, and which will overlap each other.

The PCR is a *chain reaction* because newly synthesized DNA strands will act as templates for further DNA synthesis in subsequent cycles. After about 25 cycles of DNA synthesis, the products of the PCR will include, in addition to the starting DNA, about 10^5 copies of the specific target sequence. This amount of DNA can be easily visualized as a discrete band of a specific size when submitted to agarose gel electrophoresis.

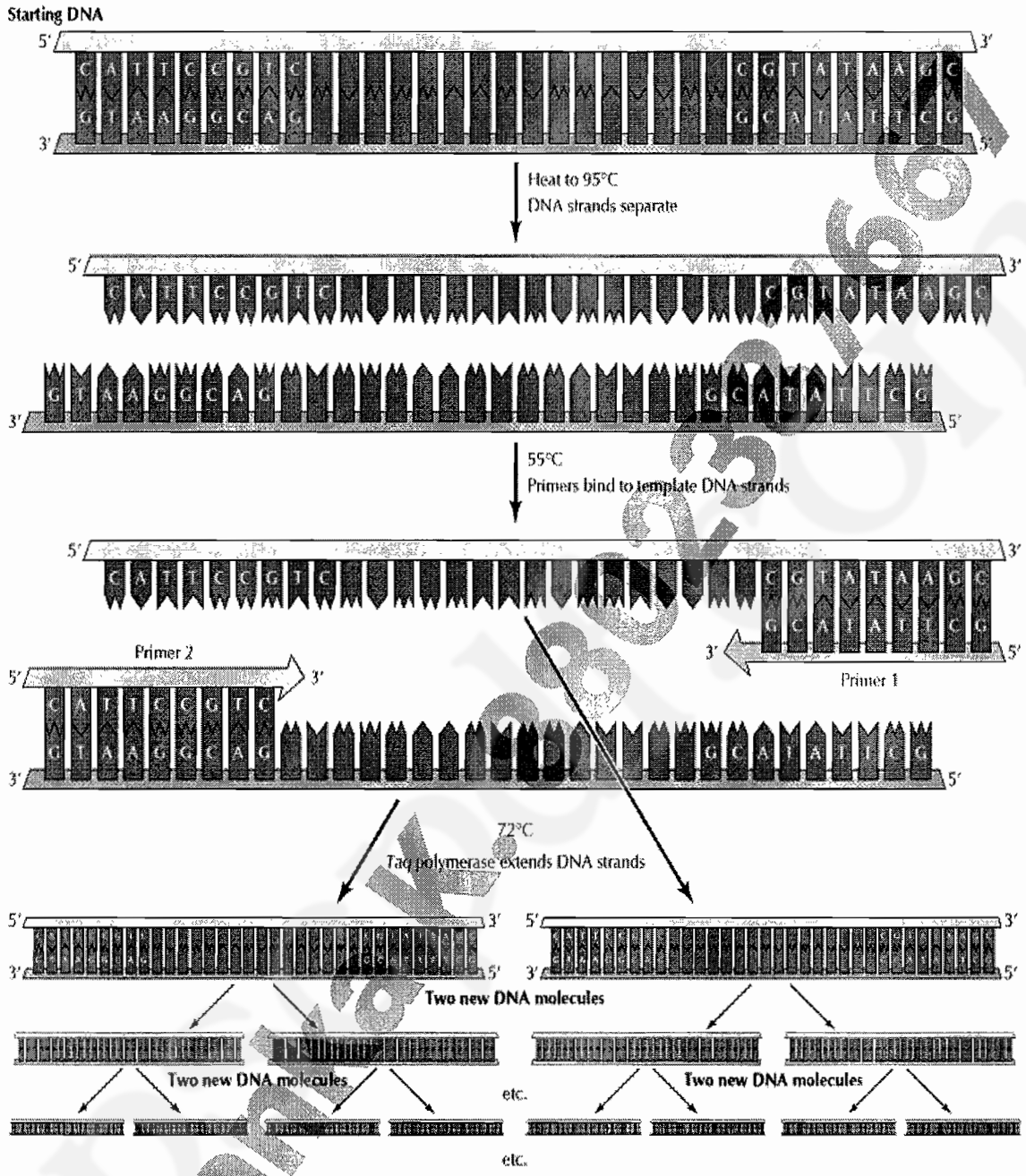


Figure 1: 1st Cycle of Amplification of DNA by PCR

A heat-stable DNA polymerase is used because the reaction involves sequential cycles composed of three steps:

1. ***Denaturation***, typically at about 93–95°C for human genomic DNA.
2. ***Reannealing*** at temperatures usually from about 50°C to 70°C depending on the T_m (T_m = Melting temperature: the temperature at which DNA strands separate) of the expected duplex (the annealing temperature is typically about 5°C below the calculated T_m).

3. ***DNA synthesis or extension***, typically at about 70–75°C. Suitably *heat-stable DNA polymerases* have been obtained from microorganisms whose natural habitat is hot springs. For example, the widely used *Taq* DNA polymerase is obtained from *Thermus aquaticus* and is thermostable up to 94°C, with an optimum working temperature of 80°C.

The region of DNA to be amplified is flanked by two sequences used to prime DNA synthesis. The starting double-stranded DNA is heated to separate the strands and then cooled to allow primers (usually oligonucleotides of 15 to 20 bases) to bind to each strand of DNA. DNA polymerase from *Thermus aquaticus* (*Taq* polymerase) is used to synthesize new DNA strands starting from the primers, resulting in the formation of two new DNA molecules. The process can be repeated for multiple cycles, each resulting in a twofold amplification of DNA.

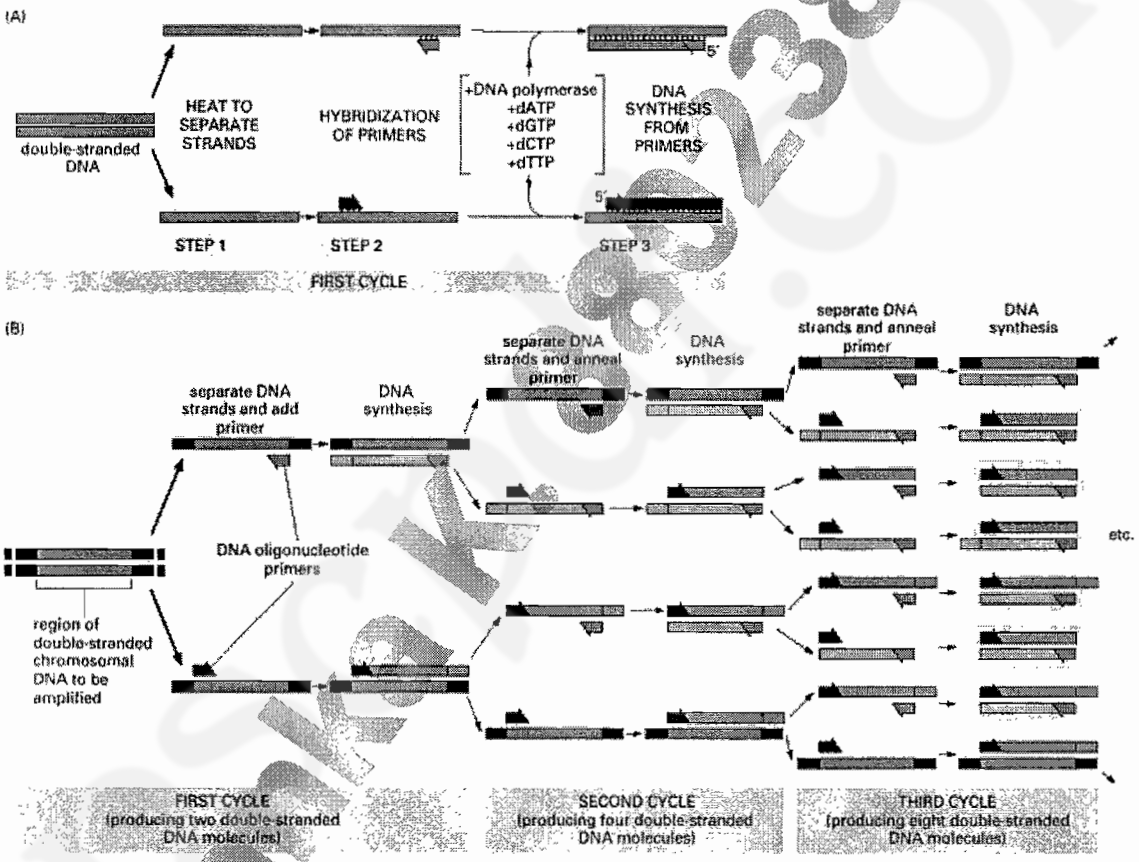


Figure 2: Amplification of DNA by Multiple Cycle of PCR

Applications

The Polymerase Chain Reaction (PCR) has found widespread application in many areas of genetic analysis. This is a list of some of these applications:

Medical applications

PCR has been applied to a large number of medical procedures:

- The first application of PCR was for *genetic testing*, where a sample of DNA is analyzed for the presence of genetic disease mutations. Prospective parents can be tested for being genetic carriers, or their children might be tested for actually being affected by a disease. DNA samples for prenatal testing can be obtained by amniocentesis, chorionic villus sampling, or even by the analysis of rare fetal cells circulating in the mother's bloodstream. PCR analysis is also essential to Pre-implantation genetic diagnosis, where individual cells of a developing embryo are tested for mutations.
- PCR can also be used as part of a sensitive test for *tissue typing*, vital to organ transplantation. There is even a proposal to replace the traditional antibody-based tests for blood type with PCR-based tests.
- Many forms of *cancer* involve alterations to oncogenes. By using PCR-based tests to study these mutations, therapy regimens can sometimes be individually customized to a patient.

Infectious disease applications

Characterization and detection of infectious disease organisms have been revolutionized by PCR:

- The Human Immunodeficiency Virus (or HIV), responsible for AIDS, is a difficult target to find and eradicate. The earliest tests for infection relied on the presence of antibodies to the virus circulating in the bloodstream. However, antibodies don't appear until many weeks after infection, maternal antibodies mask the infection of a newborn, and therapeutic agents to fight the infection don't affect the antibodies. PCR tests have been developed that can detect as little as one viral genome among the DNA of over 50,000 host cells. Thus, infections can be detected earlier, donated blood can be screened directly for the virus, newborns can be immediately tested for infection, and the effects of antiviral treatments can be quantified.
- Some disease organisms, such as that for Tuberculosis, are difficult to sample from patients and slow to be grown in the laboratory. PCR-based tests have allowed detection of small numbers of disease organisms (both live or dead), in convenient samples.

Forensic applications

The development of PCR-based *genetic (or DNA) fingerprinting* protocols has seen widespread application in forensics. In its *most discriminating form,* Genetic fingerprinting can uniquely discriminate any one person from the entire population of the world.

- Minute samples of DNA can be isolated from a crime scene, and compared to that from suspects, or from a DNA database of earlier evidence or convicts.
- PCR tests are often used to rapidly rule out suspects during a criminal investigation. Evidence from decades-old crimes can be tested, confirming or exonerating the people originally convicted.
- *Less discriminating forms* of PCR based DNA fingerprinting can help in Parental testing, where an individual is matched with their close relatives. The actual biological father of a newborn can also be confirmed (or ruled out).Similar testing can be used to confirm the biological parents of an adopted (or kidnapped) child.
- DNA from unidentified human remains can be tested, and compared with that from possible parents, siblings, or children.

Research applications

PCR has been applied to many areas of research in molecular genetics:

- PCR allows rapid production of short pieces of DNA, even when nothing more than the sequence of the two primers is known. This ability of PCR augments many methods, such as generating hybridization probes for Southern or northern blot hybridization. PCR supplies these techniques with large amounts of pure DNA.
- The task of *DNA sequencing* can also be assisted by PCR.
- Known segments of DNA can easily be produced from a patient with a genetic disease mutation.
- PCR has numerous applications to the more traditional process of DNA cloning. It can extract segments for insertion into a vector from a larger genome, which may be only available in small quantities.

FISH

Introduction

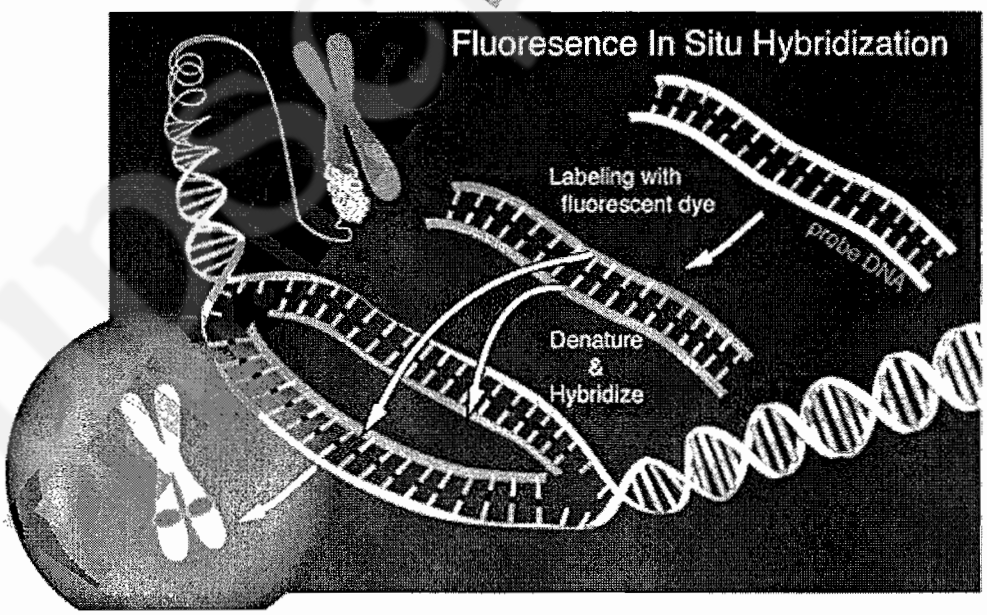
Fluorescence in situ hybridization (FISH) *is a powerful technique for detecting RNA or DNA sequences in cells, tissues, and tumors*. It is a version of hybridization analysis in which an intact chromosome is examined by probing it with a fluorescent labeled DNA molecule.

It has now become the preferred method for localization of specific nucleic acids sequences in native context as it allows very precise spatial resolution of morphological and genomic structures. The technique is rapid, simple to implement, and offers great probe stability. The genome of a particular species, entire chromosomes, chromosomal-specific regions, or single-copy unique sequences can be identified, depending on the probes used.

History: In the early versions of *in situ hybridization* the probe was *radioactively* labeled but this procedure was unsatisfactory because it is difficult to achieve both sensitivity and resolution with a radioactive label. These problems were solved in the late 1980s by the development of *non-radioactive fluorescent DNA labels*. These labels combine high sensitivity with high resolution and are ideal for *in situ hybridization*.

Principle

In principle, the technique is straightforward. A cell with intact DNA in its nucleus is treated to denature the DNA, forming single stranded regions. The *fluorescently labeled DNA probe* is added, and the single stranded probe can anneal with the corresponding sequence inside the nucleus. The *hybrid molecules* will fluoresce when the light from a fluorescent microscope excites the tag on the probe. This technique can localize the gene of interest to different areas of the nucleus or to individual chromosomes.



Specimen Types

1. ***Metaphase chromosomes***. FISH was originally used with *metaphase chromosomes*. These chromosomes, prepared from nuclei that are undergoing division, are highly condensed. With metaphase chromosomes, a fluorescent signal obtained by FISH is mapped by measuring its position relative to the end of the short arm of the chromosome. A disadvantage is that the highly condensed nature of metaphase chromosomes means that only low-resolution mapping is possible. Two markers must be at least 1 Mb apart to be resolved as separate hybridization signal. This degree of resolution is insufficient for the construction of useful chromosome maps. Therefore, the main application of metaphase FISH has been in determining the chromosome on which a new marker is located, and providing a rough idea of its map position.
2. ***Interphase chromosomes*** are more useful because this stage of the cell cycle (between nuclear divisions) is when the chromosomes are most uncondensed. Resolution down to 25 kb is possible, but chromosome morphology is lost so there are no external reference points against which to map the position of the probe. This technique is therefore used after preliminary map information has been obtained.
3. ***Fibre FISH:*** To improve the resolution of FISH to better than 25 kb it is necessary to abandon intact chromosomes and instead use purified DNA. This approach is called ***fiber-FISH***. It makes use of DNA prepared by *gel stretching or molecular (chromosome) combing* and can distinguish markers that are less than 10 kb apart. In *the process of fibre FISH*, interphase chromosomes are attached to a slide in such a way that they are stretched out in a straight line by applying mechanical shear along the length of the slide. A technique known as *chromosome combing* is increasingly used for this purpose. The extended conformation of the chromosomes allows much higher resolution - up to just a few kilobases.

Types of probes for FISH

Generally, researchers use three different types of FISH probes, each of which has a different application:

- *Locus specific probes* bind to a particular region of a chromosome. This type of probe is useful when researchers have isolated a small portion of a gene and want to determine on which chromosome the gene is located.
- *Alphoid or centromeric repeat probes* are generated from repetitive sequences found in the centromere of each chromosome.
- *Whole chromosome probes* are actually collections of smaller probes, each of which binds to a different sequence along the length of a given chromosome. Using multiple probes labeled with a mixture of different fluorescent dyes, scientists are able to label each chromosome in its own unique color. The resulting full-color map of the chromosome is known as a *spectral karyotype*. Whole chromosome probes are particularly useful for examining chromosomal abnormalities, for example, when a piece of one chromosome is attached to the end of another chromosome.

Process of FISH

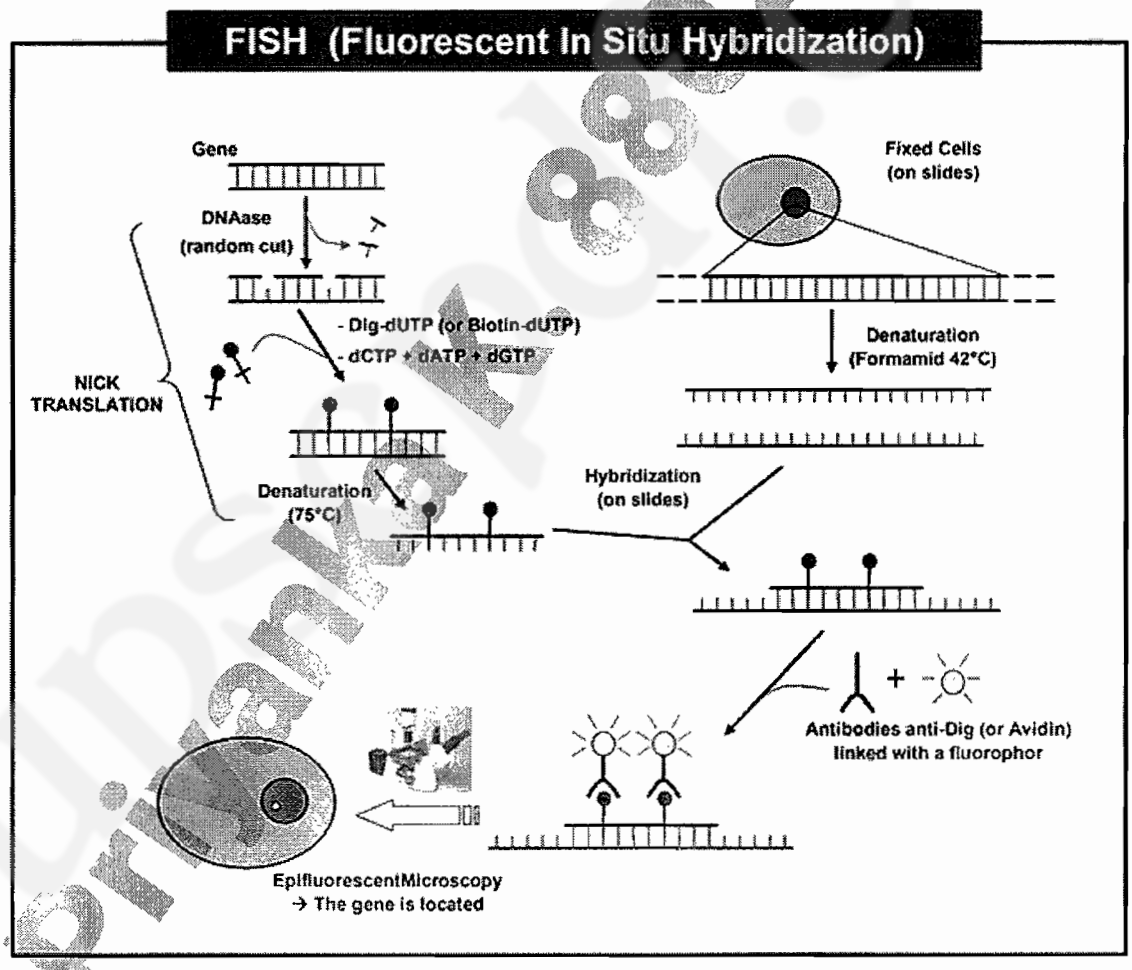
1. First, a probe is constructed. The probe must be large enough to hybridize specifically with its target but not too large to impede the hybridization process. The probe is tagged directly with fluorophores.

2. Then, an interphase or metaphase chromosome preparation is produced. The chromosomes are firmly attached to a substrate, usually glass.

3. The probe is then applied to the chromosome DNA and incubated for approximately 12 hours while hybridizing. Several wash steps remove all unhybridized or partially-hybridized probes.

4. The results are then visualized and quantified using a microscope that is capable of exciting the dye and recording images.

If the fluorescent signal is weak, amplification of the signal may be necessary in order to exceed the detection threshold of the microscope.



Applications

The use of FISH is growing rapidly in genomics, cytogenetics, prenatal research, tumor biology, radiation labels, gene mapping, gene amplification, and basic biomedical research.

- FISH has a large number of applications in *molecular biology and medical science*, including gene mapping, diagnosis of chromosomal abnormalities, and studies of cellular structure and function. Chromosomes in three-dimensionally preserved nuclei can be "painted" using FISH.
- In *clinical research*, FISH can be used for prenatal diagnosis of inherited chromosomal aberrations, postnatal diagnosis of carriers of genetic disease, diagnosis of infectious disease, viral and bacterial disease, tumor cytogenetic diagnosis, and detection of aberrant gene expression. Examples of diseases that are diagnosed using FISH include Prader-Willi syndrome, Angelman syndrome, 22q13 deletion syndrome, chronic myelogenous leukemia, acute lymphoblastic leukemia, Cri-du-chat, Velocardiofacial syndrome, and Down syndrome.
- In *laboratory research*, FISH can be used for mapping chromosomal genes, to study the evolution of genomes (***Zoo FISH***), analyzing nuclear organization, visualization of chromosomal territories and chromatin in interphase cells, to analyze dynamic nuclear processes, somatic hybrid cells, replication, chromosome sorting, and to study tumor biology.
- It can also be used in *developmental biology* to study the temporal expression of genes during differentiation and development. Recently, high resolution FISH has become a popular method for ordering genes or DNA markers within chromosomal regions of interest.

Methods of transfer of genes

Introduction

Gene transfer or DNA uptake refers to the process that moves a specific piece of DNA (usually a foreign gene ligated to a vector) into cells. *In vitro* gene transfer is the laboratory technique of transferring refined desirable genes across taxonomic boundaries into plant and animals from other plants, animals and microbes. It also involves introduction of artificial, synthetic or chimeric genes into target cells.

The directed desirable gene transfer from one organism to another and the subsequent stable integration and expression of a foreign gene into the genome is referred as genetic transformation. The transferred gene is known as *transgene,* and the organisms that develop after a successful gene transfer are known as *transgenics.* Transgenic plants are the plants that carry the stably integrated foreign genes. These plants may also be called transformed plants.

Creation of transformed lines of plants by genetic engineering techniques is a method much faster and precise than the conventional methods of breeding. In plant breeding, techniques involving gene transfer through sexual and vegetative propagation are well established but it is very time consuming, for example it usually takes 6-8 years to produce a new variety of wheat or rice by sexual propagation.

The development of gene transfer techniques in plants has been very rapid. The techniques presently available rely on *natural plant vectors* as well as *vectorless systems*, which include directed physical and chemical methods for delivering foreign DNA into plant cells.

Some important terms and expressions related to the gene transfer methods in plants

Some important terms and expressions related to the gene transfer methods in plants have been explained below before the actual discussion on gene transfer methods.

Transient and Stable Gene Expression

If the transferred DNA is expressed for only a short period of time following the DNA transfer process, this is called *transient expression.* Most of the DNA introduced into the cell by direct gene transfer methods is transient expressing type.

Stable expression occurs when DNA is integrated into the plant nuclear or plastid genomes, and expression occurs in regenerated plants and is inherited in subsequent generations. For stable transformation, the developmental potential of the transformed cells is very important. Ideally, the transformed cells should be capable of regeneration into fertile plants.

Marker Genes

Monitoring and detection of plant transformation systems in order to know whether the DNA has been successfully transferred into recipient cells is done with the help of a set of genes. The marker gene(s) is/are introduced into the vector along with the target gene. Marker genes are categorized into two types:

1. *Reporter or scoreable genes* are such genes which confer a directly detectable morphological or biochemical trait to the transformed organism.

2. *Selectable marker genes* are mainly antibiotic resistance genes (or herbicide resistance genes for plants) the presence of which can be determined by treating the transformed cells with the corresponding antibiotic or herbicide substance.

Important scorable and selectable marker genes used in plant cell transformation are tabulated below.

Table 1: Important scorable marker genes

Reporter gene	Substrate and assay	Identification
Octopine synthase (*ocs*)	Arginine + pyruvate + NADH	Electrophoresis, chromatography
Nopaline synthase (*nos*)	Arginine + ketoglutaric acid + NADH	Electrophoresis, chromatography
Chloramphenicol acetyl transferase (*cat*)	^{14}C Chloramphenicol + acetyl CoA; TLC separation	Autoradiography
β-Glucuronidase (*gus*)	Glucuronides (X-Gluc, 4-MUGluc, PNPG, NAG, REG)	Fluorescence detection, colorimetric, histochemical
Bacterial luciferase (*Lux F2*)	Decanal and $FMNH_2$	Bioluminiscence
Firefly luciferase	ATP + O_2 + luciferin	Bioluminiscence
Green fluorescent protein	No substrate required; UV light irradiation	Fluorescence detection
Anthocyanin regulatory genes	No substrate required	Visual

Table 2: Important selectable marker genes

Selectable marker gene	Substrates used for selection
Neomycin phosphotransferase (*npt*II)	G418, kanamycin, neomycin, paromycin
Hygromycin phosphotransferase (*hpt*)	Hygromycin B
Gentamycin acetyl transferase	Gentamycin
Streptomycin phosphotransferase	Streptomycin
Dihydrofolate reductase (*dhfr*)	Methotrexate
Phosphinothricin acetyltransferase (*bar*)	L-phosphinothricin (PPT), bialaphos
5-enolpyruvyl shikimate -3-phosphate (EPSP) synthase (*aroA*)	Glyphosate (roundup)
Acetolactate synthase mutant form (*als*)	Sulphonylurea, imidazolinones
Bromoxynil nitrilase (*bxn*)	Bromoxynil

The advantages of using marker genes lie in their easy assay, which requires no DNA extraction, electrophoresis, or autoradiography. Some genes are used both as selectable markers and reporter genes, whereas some are used either as selectable markers or as reporter genes.

Chimeric Gene Vectors and Choice of Promoters

In the majority of cases of gene transfer, marker genes and the genes of interest are modified prior to the transfer by different methods. These are placed under the control of different promoter, terminator, and enhancer sequences. These are called chimeric or transgene constructs, since they consist of components derived from different origins. Plants are usually transformed with relatively simple constructs in which the gene of interest is coupled with an appropriate promoter, 5' leader and a 3' terminator sequences to ensure efficient transcription, stability, and translation of mRNA.

The promoter can be of plant, viral, or bacterial origin. Some promoters confer constitutive expression, whereas others may be selected to permit tissue-specific expression or environmentally inducible expression. The promoters of bacterial origin are *lac, trp,* and *tac,* and phage promoters are T3, T7, and SP6. The cauliflower mosaic virus (CaMV) 35S RNA promoter is often used because it is a plant virus promoter, and plant viruses are dependent on transcription and translation factors of plants. It directs high levels of expression in most tissues. Others such as maize ubiquitin I promoter, *rbcS* ribulose bisphosphate carboxylase small subunit, *Adh1* alcohol dehydrogenase, *nos* nopaline synthase, and the rice actin promoter/ intron sequence are often preferred for expression in monocots.

Methods of transfer of genes in Plants

Methods for plant transformation mostly utilize:

1. Direct gene transfer methods
2. *Agrobacterium* mediated transformation
3. Virus mediated gene transfer
4. Floral dip method

Direct Gene Transfer Methods

The term direct gene transfer is used to discriminate between methods of plant transformation that rely on the use of *Agrobacterium* or viruses (indirect methods) and those that do not (direct methods). Direct gene transfer methods all rely on the delivery of large amounts of naked DNA by mechanical or chemical methods rather than biological methods.

Four important methods for direct gene transfer have been developed over the years.

1. **Particle bombardment** (biolistics) is the most important and most effective direct gene transfer method in regular use. In this technique, tungsten or gold particles are coated with the DNA that is to be used to transform the plant tissue. The particles are propelled at high speed into the target plant material, where the DNA is released within the cell and can integrate into the genome. The delivery of DNA using this technology has allowed a lot of transient gene expression but integration of the transgene also occurs infrequently.

   Practical bombardment systems were first developed in 1987 and used an explosive charge to propel DNA-coated tungsten particles. This technology was the key to cereal transformation. All the major cereals were able to be transformed, including *Golden Rice*, were produced by this method.

2. **Polyethylene glycol (PEG)-mediated transformation**: Plant protoplasts (plant cells that have had their cell walls removed) can be transformed with naked DNA by treatment with polyethylene glycol (PEG) in the presence of divalent cations (usually Calcium). The PEG and the divalent cations destabilize the plasma membrane of the plant protoplast and render it permeable to naked DNA. Once inside the protoplast the DNA enters the nucleus and integrates into the genome.

3. **The electroporation of cells** can be used to deliver DNA into plant cells and protoplasts. The vectors used can be simple plasmids; the genes of interest require plant regulatory

sequences, but no specific sequences are required for integration. Material is incubated in a buffer solution containing DNA and subjected to high-voltage electrical pulse. The DNA then migrates through high-voltage-induced pores in the plasma membrane and integrates into the genome. Electroporation has been successfully used to transform all the major cereals, particularly rice, wheat and maize. Initially, protoplasts were used for transformation, but one of the advantages of the system is that both intact cells and tissues can be used. This reduces some, but not all, of the tissue culture problems. However, the plant material used for electroporation may require specific treatments, such as pre- and postelectroporation incubations in high osmotic buffers. The efficiency of electroporation is also questionable, it is very dependent on the condition of the plant material used and the electroporation and tissue treatment conditions chosen.

4. **Silicon Carbide Fibers:** This is a simple technique for which no specialized equipment is required. Plant material (such as cells in suspension culture, embryos and embryoderived calluses) is introduced into a buffer containing DNA and the silicon carbide fibres, which is then vortexed. The fibres, which are about 0.3-0.6 □m in diameter and 10-100 □m long, penetrate the cell wall and plasma membrane, allowing the DNA to gain access to the inside of the cell.

Table 3: The relative merits of different direct methods of gene transfer

Direct gene transfer method	Comments
Particle bombardment	Very successful method. Risk of gene rearrangements and high copy number. Useful for transient expression assays
Electroporation	Transgenic plants obtained from a range of cereal crops. Low efficiency. Requires careful optimisation
DNA uptake into protoplasts	Used for all major cereal crops. Requires optimisation with a regenerable cell suspension that may not be available
Silicon carbide fibres	Requires regenerable cell suspensions. Transgenic plants obtained from a number of species

Other, less-reproducible direct methods include:

1. Laser-mediated uptake of DNA
2. Microinjection
3. Ultrasound and
4. *In planta* exogenous application.

Agrobacterium Mediated Gene Transfer

Agrobacterium is a genus of soil borne Gram-negative bacteria that uses horizontal gene transfer to cause tumors in plants. *Agrobacterium tumefaciens* is the most commonly studied species in this genus. *A. tumefaciens* causes crown-gall disease in plants. The disease is characterised by a tumour-like growth or gall on the infected plant, often at the junction between the root and the shoot. Tumors are incited by the conjugative transfer of a DNA segment (**T-DNA**) from the bacterial tumour-

inducing (Ti) plasmid. The closely related species, *A. rhizogenes*, induces root tumors, and carries the distinct Ri (root-inducing) plasmid.

Agrobacterium is unique for its ability to transfer DNA between itself and plants, and for this reason it has become an important tool for plant improvement by genetic engineering. Marc Van Montagu and Jozef Schell at the University of Ghent (Belgium) discovered the gene transfer mechanism between *Agrobacterium* and plants, which resulted in the development of methods to alter *Agrobacterium* into an efficient delivery system for gene engineering in plants.

A modified Ti or Ri plasmid can be used. The plasmid is 'disarmed' by deletion of the tumor inducing genes; the only essential parts of the T-DNA are its two small (25 base pair) border repeats. The genes to be introduced into the plant are cloned into a plant transformation vector that contains the T-DNA region of the disarmed plasmid, together with a selectable marker (such as antibiotic resistance) to enable selection for plants that have been successfully transformed. Plants are grown on media containing antibiotic following transformation, and those that do not have the T-DNA integrated into their genome will die.

The method of *Agrobacterium* mediated gene transfer is described below for tobacco.

Tobacco is a relatively easy plant to transform with *Agrobacterium* and provides a good introduction to the use of *Agrobaeterium* in plant transformation.

Several factors have to be considered in the design and implementation of any plant transformation study:

1. **The plant tissue to be transformed.** The purpose of most plant transformation experiments in plant biotechnology is to produce whole transgenic plants. The explant used in transformation experiments must therefore be capable of producing whole plants by re-generation, and should contain a high proportion of cells that are competent for transfor-mation.

2. **The vector used to deliver the transgene into the genome of the plant.** The construction of vectors for use in *Agrobacterium*-mediated transformation have some key points:

   a. They are derivatives of, or are based on, the naturally occurring *Ti* plasmid.

   b. They are extensively modified so that most of the features of a natural *Ti* plasmid are removed, only the left- and right-border sequences being used to ensure transfer of the T-DNA region between them.

   c. The vector also contains a selectable marker on the T-DNA so that transformed plants can be identified.

3. **The strain of *Agrobacterium* used.** Several widely used strains of *Agrobacterium* are available for plant transformation experiments. For the transformation of crops that, like tobacco, are amenable to *Agrobacterium*-mediated transformation, the choice of strain is not critical to the success of the experiment. However, for more recalcitrant plant species, the choice of strain is a major factor contributing to the success or failure of the experiment.

The basic protocol used for any Agrobacterium-mediated transformation experiment can be illustrated with the following example of tobacco transformation.

a. Suitable plant tissue, to be used as a source of explants, is removed from the donor plant and sterilised (if it is not from a sterile plant). For tobacco transformation, leaves are ideal.

b. The leaf tissue is cut into small pieces (using a scalpel or cork-borer) and placed into a culture of *Agrobacterium* (which contains the vector) for about 30 minutes, a process known as **co-cultivation**. During this incubation, the bacteria attach to the plant cells. The explants are subsequently removed from the bacterial culture, excess bacterial culture blotted off, and then placed on to solid nutrient medium that contains no selective agent.

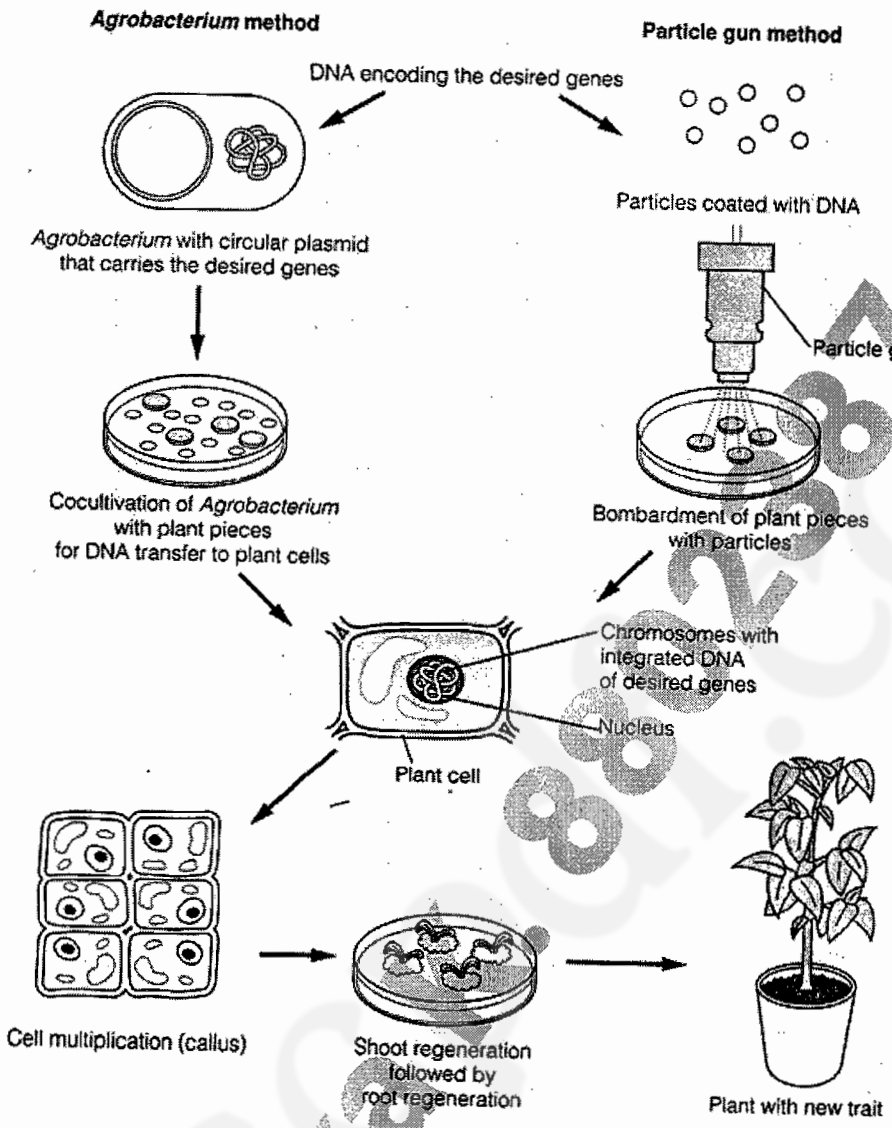
c. The incubation of the explants with *Agrobacterium* is allowed to continue for 2 days to allow transfer of the T-DNA to the plant cells.

d. The explants are removed from the medium and washed in an antibiotic solution (such as Cefotaxime) that kills *Agrobacterium* cells.

e. The explants are transferred to fresh solid medium. This medium is supplemented with a selective agent (often Kanamycin, but this will depend on which selectable marker gene is present in the T-DNA of the vector) to prevent the growth of non-transformed plant cells. It also contains Cefotaxime (to prevent the growth of any Agrobacterium that were not killed by the initial treatment with Cefotaxime).

f. An auxin and a cytokinin are also included to encourage regeneration by organogenesis. The relatively high cytokinin to auxin ratio promotes shoot formation from the explants. These shoots can be rooted by placing them on solid medium containing a high auxin to cytokinin ratio.

A comparison of *Agrobacterium* method and Particle Bombarding method of gene transfer is given below.



Agrobacterium does not usually infect monocot plant species, but very effective for dicot species. *Agrobacterium* is listed as being the original source of genetic material that was transferred to these food plants:

1. Soybean
2. Cotton
3. Corn
4. Sugar Beet
5. Alfalfa
6. Wheat
7. Rapeseed Oil (Canola)

8. Creeping bentgrass (for animal feed)

Virus Mediated Gene Transfer

Viruses provide natural examples of genetic engineering, since viral infection of a cell results in the addition of new genetic material which is expressed in the host. In current practices, there is frequent use of Gemini viruses and Caulimoviruses (CaMV) as gene vectors for plants.

Use of Gemini viruses as Cloning Vector - One advantage this group of viruses does have is that they contain DNA which, although single stranded, appears to replicate via a double-stranded intermediate, which would make in vivo manipulation in bacterial plasmids more convenient. The virus group is known to infect a wide range of crop plants, including monocots and dicots. Attempts are underway to develop wheat dwarf geminiviruses as vectors.

Use of CaMV as Vector - This virus helps researchers to introduce genes into plants in an effective way that would promotes their spread and expression. One useful feature of CaMV is that the naked DNA is, infective, being able to enter plant cells directly if rubbed onto a leaf with a mild abrasive. Once inside the cells, the DNA is replicated and encapsidated within virus particles, which then invade the rest of the plant. Although the CaMV DNA does not become integrated into the chromosomal DNA and is therefore not certain to be handed on to all cells during cell division, its spread throughout the plant means that transformed plants can be effectively cloned by vegetative propagation.

Floral Dip Method

One of the most promising methods, that was developed using a member of the genus *Arabidopsis* as the target plant, is the floral dip. This is an extremely simple method in which plants with young flowers are dipped (with or without a vacuum being applied) into a culture of *Agrobacterium* which also contains a surfactant. The plant is subsequently allowed to set seed, whereupon a small proportion of the seeds produced are transgenic. Although the efficiency of this technique is very low (at present, at least) the vast number of seeds produced results in an acceptable overall transformation efficiency. This, or similar techniques have been applied successfully to other plant species, including alfalfa and some brassics.

Transgenic Crops

Transgenic plants: An overview

Plants in which one or more genes from any other plant, animal or micro-organism have been incorporated in their genome employing technique of genetic engineering are referred to as **transgenic plants**. A popular term for transgenic plants is **genetically modified plants**. In case of crops, it is termed **genetically modified crops or GM crops.** The food prepared from GM crops is called GM food or genetically modified food. GM foods differ from the produce of traditionally produced varieties in several aspects:

- It contains the protein produced by the transgene in question, e.g., ***cry* protein** in the case of insect-resistant varieties. In all cases, it contains the novel trait for which the transgenic plant was created.
- It contains the enzyme produced by the antibiotic resistance gene that was used as marker during gene transfer by genetic engineering.
- It contains the antibiotic-resistant gene itself.

Transgenic plants find use in agriculture, industry, medicine and environmental cleanup.

Potential amenable characters which can be introduced through gene transfer to crop plants are:

- Disease resistance
- Delayed ripening of fruits
- Herbicide tolerance
- Insect pest resistance
- Drought and stress tolerance
- Production of foreign proteins
- Enhanced nitrogen availability
- Expression of high value products in seeds
- Efficient photosynthesis
- Production of male sterility
- Production of secondary metabolites
- Improvement in vitamin A content
- Improvement in iron content in crops like rice
- Production of transgenics that can be used as edible vaccines
- Improvement in the content and composition (fatty acid composition) of edible oils in rapeseeds and other oilseed crops
- Improvement in the architecture, colour, fragrance and vase life of flowers of commercial value.

Some important traits of benefit are explained briefly below.

Beneficial Product Traits	Crops
Bt crops are protected against insect damage and reduce pesticide use. Plants produce a protein—toxic only to certain insects—found in *Bacillus thuringiensis*.	Corn, cotton, potatoes **Future:** sunflower, soybeans, canola, wheat, tomatoes
Herbicide-tolerant crops allow farmers to apply a specific herbicide to control weeds without harm to the crop. They give farmers greater flexibility in pest management and promote conservation tillage.	Soybeans, cotton, corn, canola, rice **Future:** wheat, sugar beets
Disease-resistant crops are armed against destructive viral plant disease with the plant equivalent of a vaccine.	Sweet potatoes, cassava, rice, corn, squash, papaya **Future:** tomatoes, bananas
High-performance cooking oils maintain texture at high temperatures, reduce the need for processing, and create healthier food products. The oils are either high oleic or low linoenic. In the future, they will also be high stearate.	Sunflower, peanuts, soybeans
Healthier cooking oils have reduced saturated fat.	Soybeans
Delayed-ripening fruits and vegetables have superior flavor, color, and texture; are firmer for shipping; and stay fresh longer.	Tomatoes **Future:** raspberries, strawberries, cherries, tomatoes, bananas, pineapples
Increased-solids tomatoes have superior taste and texture for processed tomato pastes and sauces.	Tomatoes
rBST is a recombinant form of a natural hormone, bovine somatotropin, which causes cows to produce milk. rBST increases milk production by as much as 10–15%. It is used to treat over 30% of U.S. cows.	rBST (milk production)
Food enzymes, including a purer, more soluble form of chymosin used to curdle milk in cheese production, are used to make 60% percent of hard cheeses. Replaces chymosin of rennet from slaughtered calves' stomachs.	Chymosin (in cheese)—the *first* biotechnology product in food
Nutritionally enhanced foods will offer increased levels of nutrients, vitamins, and other healthful phytochemicals. Benefits range from helping developing nations meet basic dietary requirements to boosting disease-fighting and health-promoting foods.	**Future:** protein-enhanced sweet potatoes and rice; high-vitamin-A canola oil; increased antioxidant fruits and vegetables

The table below depicts the crops that were genetically manipulated for some desirable trait and released on commercial basis.

Crop	*Gene (s) Introduced*	*New/Improved character*	*Developer*
Canola	Thioesterase	High laurate oil	Calgene
	EPSP synthetase	Weed control	Monsanto
	PAT & Barnase/ Barstar	Weed control and Hybrid production	AgrEvo
Corn	EPSP synthetase	Weed control	Monsanto
	Bt CrylA(b)	Insect resistance	Monsanto, Ciba-Geigy Northrup King
	Bt CrylA(c)	Insect resistance	Dekalb Genetics
Cotton	Acetolactate synthetase	Weed control	DuPont
	Nitrilase	Weed control	Calgene
	EPSP synthetase	Weed control	Monsanto
	Bt CrylA(c)	Insect control	Monsanto
Papaya	Coat protein	Virus resistance	Univ. Hawaii & Cornell Univ.
Potato	Bt CryIIIA & Coat protein	Insect & virus control	Monsanto
Soybean	EPSP synthetase	Weed control	Monsanto
Sugarbeet	Gmfad2-1	Improved oil	DuPont
Tomato	Bt CrylA(c)	Insect control	Monsanto
	Antisense PG	Delayed ripening	Calgene

The list of gene modifications that directly aid consumers will only grow. The future targets of plant transgenics are enumerated in the table below.

Goal	Application
Salinity tolerance	Increased crop yield in areas affected by salinity (e.g. in long-term irrigation)
Drought tolerance	Increased crop yield in marginal, semi-arid zones
Waterlogging tolerance	Improved survival in temporary flooding
Enhanced flavor, storage and properties	Improved consumer acceptance; decreased losses; decreased energy inputs to processing or storage; enhanced product value or usefulness
Enhanced amino acid content	Dietary improvement and health
Antibody and pharmaceutical production	Less energy input and cost than use of animal cell culture; less use of animals
Improved disease resistance	Reduced pesticide inputs; increased yields mean population can be fed using smaller land area

Areas of concern regarding transgenic plants

There are several areas of concern regulating the use of genetically modified crop foods. This includes toxicity, allergenicity, cardiogenicity, food intolerance and nutritional value. While the supporters of the technology argue that the food produced through biotechnology are just as safe, if not safer than conventionally produced foods, because they are subjected to highly rigorous testing.

Most of the environmental concerns about GM technology in plants have been derived from the possibilities of gene flow to close relatives of transgenic plants creating super weeds or causing gene pollution in other crops.

Various arguments against herbicide-tolerant transgenics in crop plants have come up lately:

1. Use of herbicide-tolerant transgenic crops can lead to transfer of herbicide tolerance genes to sexually compatible wild relatives or weeds, which can be a major potential threat to environment.
2. Transgenic crops can create "super weeds".
3. It would actually increase the dependence on a few herbicides rather than reducing herbicide usage.
4. It may increase the problem of weed control if weeds develop resistance to such herbicides through gene flow from transgenic crops.
5. Herbicide tolerance is being sought not only for environmentally comparatively acceptable herbicides but also for older, more toxic and persistent products.
6. Nonchemical means of weed control, such as crop rotation, dense plantings, cover cropping, ridge tillage, and others, however labour-intensive for farmers, are preferable' than the use of any herbicide at all. Gene flow is the primary risk in releasing transgenic plants.

As of now, except Bt cotton, no other transgenic or genetically modified (GM) crop has yet been commercialized in India, though extensive efforts are in progress at different laboratories across the country to develop the GM food crop.

Biosafety aspects

The concept of Biosafety

Biosafety is prevention of large-scale loss of biological integrity, focusing both on ecology and human health. The international Biosafety Protocol deals primarily with the agricultural definition. In agriculture, biosafety mainly implies reducing the risk of alien viral or transgenic genes and reducing the risk of food bacterial contamination.

In modern context, Biosafety is used to describe efforts to reduce and eliminate the potential risks resulting from biotechnology and its products. It has similarly been defined as "**the avoidance of risk to human health and safety, and to the conservation of the environment, as a result of the use for research and commerce of infectious or genetically modified organisms**" (Zaid, 2001). Relevant scientific disciplines that address biosafety issues include molecular biology, plant breeding, genetics, plant pathology, agronomy, weed science, entomology and ecology, among others.

The potential risks

The potential risks resulting from plant biotechnology and its products are briefly described below.

1. **Risks for animal and human health:** Toxicity & food/feed quality/safety; allergies; pathogen drug resistance (antibiotic resistance), impact of selectable marker

2. **Risks for the environment:** Persistency of gene or transgene (volunteers, increased fitness of GM crop, invasiveness) or of transgene products (accumulative effects); susceptibility of non-target organisms; change in use of chemicals in agriculture; unpredictable gene expression or transgene instability (gene silencing); environmentally-induced (abiotic) changes in transgene expression; ecological fitness; changes to biodiversity (interference of tri-trophic interactions); impact on soil fertility/soil degradation of organic material

3. **Horizontal gene transfer:** Genetic pollution through pollen or seed dispersal & horizontal gene transfer (transgene or promoter dispersion); transfer of foreign gene to micro-organisms (DNA uptake) or generation of new live viruses by recombination (transcapsidation, complementation, etc.)

4. **Risks for agriculture:** Resistance/tolerance of target organisms; weeds or superweeds; alteration of nutritional value (attractiveness of the organism to pests); change in cost of agriculture; pest/weed management; unpredictable variation in active product availability; loss of familiarity/changes in agricultural practise

5. **General concerns:** Detection and analytical methods; ethical issues (eg. labelling); substantial equivalence; risk assessment/ risk management; general biosafety; public attitudes, perception; legislation (incl. liability & redress); monitoring; socio-economics (eg. situation of poor farmers in developing countries); IPR (Intellectual Property Rights); GM traceability / commodity segregation.

Efforts in the direction of biosafety

The Cartagena Protocol on Biosafety

The Cartagena Protocol on Biosafety to the CBD is an international treaty governing the movements of living modified organisms (LMOs) resulting from modern biotechnology from one country to another that may have adverse effect on the conservation and sustainable use of biological diversity. Living Modified Organisms are GMOs resulting from modern biotechnology which is capable of growing, and typically refers to agricultural crops. It was adopted on 29 January 2000 as a supplementary agreement to the Convention on Biological Diversity and entered into force on 11 September 2003. The Protocol applies to the transboundary movement, transit, handling and use of all such LMOs. It establishes an advance informed agreement (AIA) procedure for ensuring that countries are provided with the information necessary to make informed decisions before agreeing to the import of such organisms into their territory. It contains the precautionary approach of the Rio Declaration on Environment and Development. The Protocol also establishes a Biosafety Clearing-House (BCH) to facilitate the exchange of information on living modified organisms and to assist countries in the implementation of the Protocol.

India's Biosafety and Recombinant DNA Guidelines (1990)

India's Biosafety and Recombinant DNA Guidelines (1990) fall under the Environment (Protection) Act of 1986. In 1994, after India signed the Biodiversity Convention, the DBT revised its earlier guidelines to accommodate the safe handling of GMOs in research, application and technology transfer. This includes the large scale production and deliberate release of GMOs plants, animals and products into the environment. Guidelines are also provided for the shipment and importation of GMOs for laboratory research and field trials.

Genetic Engineering Appraisal Committee (GEAC)

The Genetic Engineering Appraisal Committee (GEAC) is a statutory body established by Ministry of Environment, Forest and Climate Change under the provisions of Environment (Protection) Act, 1986 (Rules, 1989). It is the apex body which regulates manufacturing, use, import, export and storage of:

- hazardous micro-organisms
- genetically engineered organisms
- genetically engineered cells

Responsibilities

- Approval of activities involving large-scale use of hazardous living micro-organisms and recombinants in research and industrial production **from the environmental perspective**.
- Approval of proposals relating to release of genetically engineered organisms and products into the environment, including experimental field trials.
- Approval of proposals involving the use of living modified organism falling in the risk category III and above in the manufacture/import of recombinant pharma products
- Approval of proposals where the end product of the recombinant pharma product is a living modified organism

The most recent GEAC was instituted in March 2013 and the tenure of the GEAC is for 3 years.

In India, a number of research projects involving *Genetically Modified Organisms* (GMOs), with both contained and field trials, are already in progress. This points to the urgency of assessing the effectiveness and enforcement of biosafety regulations in the country.